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Distinctive characteristics and functions of multiple mitochondrial Ca^{2+} influx mechanisms

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Intracellular Ca^{2+} is vital for cell physiology. Disruption of Ca^{2+} homeostasis contributes to human diseases such as heart failure, neuron-degeneration, and diabetes. To ensure an effective intracellular Ca^{2+} dynamics, various Ca^{2+} transport proteins localized in different cellular regions have to work in coordination. The central role of mitochondrial Ca^{2+} transport mechanisms in responding to physiological Ca^{2+} pulses in cytosol is to take up Ca^{2+} for regulating energy production and shaping the amplitude and duration of Ca^{2+} transients in various micro-domains. Since the discovery that isolated mitochondria can take up large quantities of Ca^{2+} approximately 5 decades ago, extensive studies have been focused on the functional characterization and implication of ion channels that dictate Ca^{2+} transport across the inner mitochondrial membrane. The mitochondrial Ca^{2+} uptake sensitive to non-specific inhibitors ruthenium red and Ru360 has long been considered as the activity of mitochondrial Ca^{2+} uniporter (MCU). The general consensus is that MCU is dominantly or exclusively responsible for the mitochondrial Ca^{2+} influx. Since multiple Ca^{2+} influx mechanisms (e.g. L-, T-, and N-type Ca^{2+} channel) have their unique functions in the plasma membrane, it is plausible that mitochondrial inner membrane has more than just MCU to decode complex intracellular Ca^{2+} signaling in various cell types. During the last decade, four molecular identities related to mitochondrial Ca^{2+} influx mechanisms have been identified. These are mitochondrial ryanodine receptor, mitochondrial uncoupling proteins, LETM1 (Ca^{2+}/H^{+} exchanger), and MCU and its Ca^{2+} sensing regulatory subunit MICU1. Here, we briefly review recent progress in these and other reported mitochondrial Ca^{2+} influx pathways and their differences in kinetics, Ca^{2+} dependence, and pharmacological characteristics. Their potential physiological and pathological implications are also discussed.

mitochondrial calcium channels, calcium transport, mitochondria, heart, ryanodine receptor

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It was discovered five decades ago that isolated mitochondria can rapidly take up a large quantity of Ca^{2+} in a mitochondrial membrane potential-dependent manner [1–3]. Since then substantial studies have demonstrated that the function of mitochondria is more than just a Ca^{2+} buffering system [4–8]. The Ca^{2+} taken up by mitochondria stimulates the activity of Krebs cycle and oxidative phosphorylation, and ultimately the ATP synthesis [9,10]. Moreover, by taking up Ca^{2+}, mitochondria shape the amplitude and duration of Ca^{2+} transients in subcellular micro-domains, which are critical for regulating the activity of numerous Ca^{2+} binding proteins.

The driving force of mitochondrial Ca^{2+} uptake is determined by extra mitochondrial Ca^{2+} concentrations, mitochondrial inner membrane potential, and Ca^{2+} concentrations in mitochondrial matrix. At local micro-domains such as plasma membrane and endoplasmic/sarcoplasmic reticulum (ER/SR), Ca^{2+} concentrations fall off steeply from several hundreds micro-molar ($\mu mol\ L^{-1}$) at the mouth of the open Ca^{2+} channel and reach a few $\mu mol\ L^{-1}$ within just few

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tens of nanometer (nm) away from the channel [11]. Mitochondria localized at close proximity to these intracellular Ca^{2+} stores or plasma membrane Ca^{2+} channels sense and respond to the Ca^{2+} transients by taking up Ca^{2+} so that the Ca^{2+} concentrations can reach up to μmol L^{-1} ranges in matrix when the extra mitochondrial Ca^{2+} concentrations are high [11]. This physiological increase in mitochondrial Ca^{2+} serves as a key signal for regulating mitochondrial activities. However, non-physiological Ca^{2+} overload depolarizes mitochondria by opening mitochondrial permeability transition pores (mPTP), which causes cell death [12,13]. It is particularly remarkable to appreciate the dynamic nature of mitochondrial Ca^{2+} uptake in excitable tissues such as heart and neuron, because the frequency of physiological Ca^{2+} oscillations in cytosol is in the range of 1 to 100 Hz. Therefore, mitochondria must be equipped with Ca^{2+} transport mechanisms that are capable of rapidly taking up cytosolic Ca^{2+} in order to encode the high frequencies of cytosolic Ca^{2+} pulses into cellular ATP and Ca^{2+} regulation.

For the past five decades, extensive studies have been focused on the identification and characterization of the ion channels in the inner mitochondrial membrane that dictate Ca^{2+} transport into mitochondria [12,14]. Initially mitochondrial Ca^{2+} uniporter (MCU), which permits transport of the ion down its electrochemical gradient, is thought to be the single mechanism for mitochondrial Ca^{2+} uptake. This idea is supported by the fact that the nonspecific inhibitors, ruthenium red and lanthanides, almost completely blocked mitochondrial Ca^{2+} uptake. However, further studies have demonstrated that several other pathways exist for mitochondrial Ca^{2+} influx. They are rapid mode (RaM) of mitochondrial Ca^{2+} transport [6,7,15,16], mitochondrial ryanodine receptor (mRyR) [17,19], uncoupling proteins 2 and 3 [20], and Letm1 mitochondrial Ca^{2+}/H^{+} antiporter [21] (Figure 1). These pathways exhibit differences from that of MCU in kinetics, Ca^{2+} dependence, and pharmacological properties.

The balance between Ca^{2+} influx and efflux across mitochondrial inner membrane establishes mitochondrial Ca^{2+} homeostasis. The mitochondrial Na^{+}/Ca^{2+} exchanger (NCX) normally serves as a major Ca^{2+} efflux mechanism because of an inwardly directed Na^{+} electrochemical gradient [22]. In addition, the transient opening of mPTP represents another important mechanism for Ca^{2+} release from mitochondria in physiological conditions. However, the molecular identity of mPTP still remains unknown [12]. This review focuses on the mitochondrial Ca^{2+} influx mechanisms.

1 Mitochondrial Ca^{2+} uniporter (MCU)

MCU is a ruthenium-sensitive Ca^{2+} channel existing in mitochondrial inner membrane. MCU facilitates the Ca^{2+} transport down its electrochemical gradient without coupling Ca^{2+} transport with the transport of other ions [4,23]. MCU is highly selective for Ca^{2+} but other cations can also be transported with different permeability: $Ca^{2+}>Sr^{2+}>$

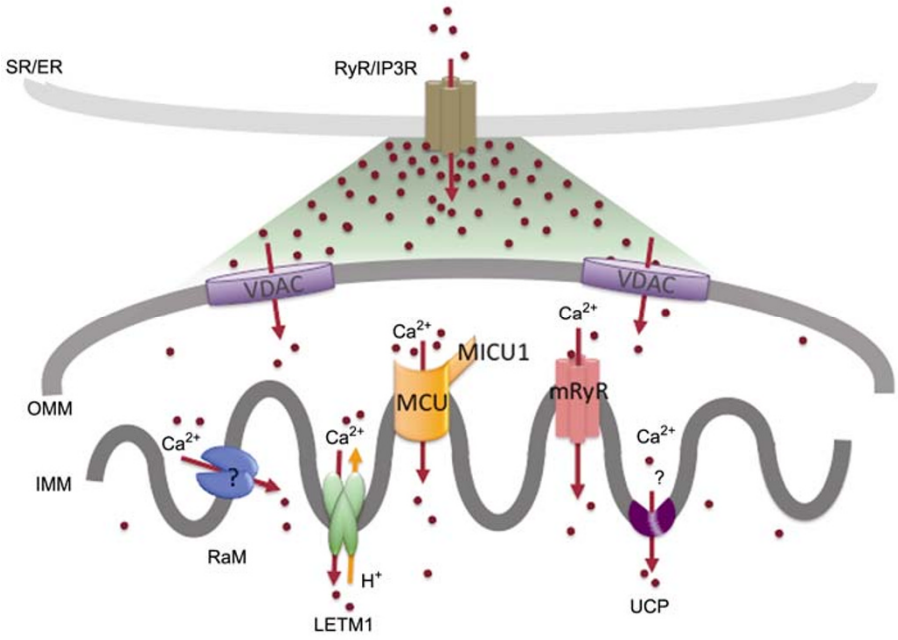

Figure 1 Ca^{2+} uptake mechanisms in the inner membrane of mitochondria. SR/ER, sarco/endoplasmic reticulum; RyR/IP3R, ryanodine/IP3 receptors; VDAC, voltage dependent anion-selective channel; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; MCU, mitochondrial Ca^{2+} uniporter; MICU1, mitochondrial calcium uptake 1; mRyR, mitochondrial ryanodine receptor; RaM, rapid mode uptake; UCP, uncoupling proteins; LETM1, mitochondrial Ca^{2+}/H^{+} exchanger.

$Mn^{2+}>Ba^{2+}>La^{3+}$ [24–27].

The most commonly used inhibitor of MCU is ruthenium red, which blocks mitochondrial Ca^{2+} uptake [28]. Besides ruthenium red, several drugs have been found inhibiting the activity of MCU [29–32]. On the other hand, inorganic phosphate (Pi) and spermine activate MCU and facilitate Ca^{2+} transport. Pi decreases free Ca^{2+} concentrations in mitochondrial matrix by precipitating Ca^{2+}, which favors additional Ca^{2+} uptake. Spermine affects Ca^{2+} uptake in a Ca^{2+} concentration-dependent manner [33]. It facilitated the uptake when Ca^{2+} concentrations were lower than 4.5 μmol L^{-1} while inhibited Ca^{2+} uptake at higher Ca^{2+} concentrations [33]. In spite of its critical importance in Ca^{2+} uptake, little is known about how MCU is regulated. Recent studies indicate that the activity of MCU is regulated by cytosolic Ca^{2+} in a calmodulin-dependent manner [34,35].

The biophysical properties of mitochondrial Ca^{2+} uptake have been extensively characterized. Recently, patch-clumping of whole mitoplasts from COS-7 cells recorded a Ca^{2+} channel that accounted for the properties of MCU [36]. This channel, named MiCa, is highly Ca^{2+} selective (affinity<2 nmol L^{-1}). It has enormous Ca^{2+} transport capacity with half saturation at 20 mmol L^{-1} Ca^{2+} concentration ($[Ca^{2+}]$). MiCa showed inwardly rectifying current with the voltage ramped from −160 to +80 mV. The current gradually increased when the $[Ca^{2+}]$ rose from 20 to 100 μmol L^{-1}, which is comparable to the micro-domains $[Ca^{2+}]$ at ER/SR or cytoplasmic membrane where Ca^{2+} is released [37–40]. At 100 μmol L^{-1} $[Ca^{2+}]$, the current density reached (55±19) pA pF^{-1} (at −160 mV). The amplitude of Ca^{2+} current through this channel showed saturation at ~105 mmol L^{-1} $[Ca^{2+}]$. Similar to MCU, ruthenium red inhibited this current with IC_{50} of 2 nmol L^{-1}. Moreover, it had identical relative divalent ion conductance to that of MCU. This finding challenges the well-established view that the relationship between the initial rates of Ca^{2+} uptake by MCU and the extra mitochondrial $[Ca^{2+}]$ is sigmoidal with a Hill coefficient of 2 and a half maximal concentration of 1–189 μmol L^{-1}.

Recently, the long sought mystery about the molecular identity of MCU is just unveiled [41,42]. MICU1, which contains a Ca^{2+} binding EF-hand, is first found to regulate MCU [43] but not directly participates in channel pore formation [44]. Using whole-genome phylogenetic profiling, genome-wide RNA co-expression analysis, and organelle-wide protein coexpression analysis, a transmembrane protein previously identified as CCDCA109A is predicted as functionally related to MICU1 and a candidate for MCU. Indeed, silencing of this gene abolished histamine-induced mitochondrial Ca^{2+} uptake in HeLa cells and isolated liver mitochondrial preparation. Topology analysis and computational predictions indicate that this protein has two predicted transmembrane domain, a linker facing matrix, and N- and C-terminus facing to the intermembrane space. Among the four negative charges, E572, S259, D261, E264, in this linker, the mutations of the three negative charges E572, D261, and E264 to alanine significantly reduced mitochondrial Ca^{2+} uptake. The mutation of S259A did not abolish mitochondrial Ca^{2+} uptake but the mitochondrial Ca^{2+} uptake was not inhibited by Ru360, thus considered as Ru360 binding site. MCU may constitute a large molecular complex (~450 kD) in the inner membrane since the disappearance of this complex silencing MCU. Same protein (~40 kD) was identified as MCU by another group and they confirmed that reconstitution of this protein show 6–7 pS single channel activity at negative voltages and the mutation of two negatively charged glutamate in the putative pore region (D260Q and E263Q) abolished the channel activity.

In summary, the MCU appears to be protein complexes consist at least two proteins; one forms the channels and the other involves in regulating the channel activities. The recent discoveries of MCU molecular identity will open up a new avenue of research that will eventually elucidate the structure and function of this key mitochondrial Ca^{2+} uptake mechanism.

2 Rapid mode (RaM) uptake

MCU was thought to be the only mechanisms for mitochondrial Ca^{2+} uptake until the discovery of rapid mode uptake. It is found that in response to cytosolic Ca^{2+} pulse like in hormone stimulation, mitochondrial Ca^{2+} concentrations ($[Ca^{2+}]_m$) go up very rapidly [39,45,46]. RaM was found first in isolated liver mitochondria as a kinetic mode which takes up Ca^{2+} approximately 1000 times faster than that via MCU [6,15,7,16].

At higher extra mitochondrial $[Ca^{2+}]$, Ca^{2+} uptake is mediated by both MCU and RaM. However, MCU does not transport Ca^{2+} when the $[Ca^{2+}]$ is below its threshold [15]. When $[Ca^{2+}]$ is below 200 nmol L^{-1}, Ca^{2+} is exclusively taken up via RaM with millisecond time scale, which completed in less than 30 ms. However, RaM also undergoes rapid inactivation at >200 nmol L^{-1} $[Ca^{2+}]$. By keeping extra mitochondrial $[Ca^{2+}]$ low (<100 nmol L^{-1}), the activity of RaM is very rapidly recovered (the reset time is less than 0.75 s).

The titration experiments showed that RaM was less sensitive to ruthenium red than MCU. The amount of ruthenium red necessary to inhibit RaM was over an order of magnitude more than that required for the inhibition of MCU (0.1 nmol L^{-1}). At similar concentrations of a known activator of MCU, spermine (0.1 mmol L^{-1} and higher), there were three times more increases of RaM than MCU. More interestingly, cyclosporine A which prevents mPTP opening had no effect on RaM suggesting a different regulatory mechanism [47,48].

Interestingly, the RaM in heart mitochondria had some different characteristics from those of liver in terms of acti-

vation and inhibition [16]. The reset time was longer (>60 s) and it was less sensitive to the inhibition by ruthenium red. Moreover, ATP and GTP activated RaM in liver but not in heart. The heart RaM is activated by ADP and inhibited by AMP.

So far, RaM is only recognized as a kinetics mode for Ca^{2+} uptake by mitochondria with unknown molecular identity. It will be interesting to find out whether RaM shares similar molecular identity with other mitochondrial Ca^{2+} influx mechanisms but shows a different kinetic mode.

3 Mitochondrial ryanodine receptor (mRyR)

The ryanodine receptor, which also rapidly takes up Ca^{2+}, was discovered in heart mitochondria by our group [19]. Many approaches were used to confirm the molecular identity of the channel, which excluded the possibility that it was the contaminant from SR. Immuno-gold particle and electron microscopy analysis showed that mRyR in isolated heart mitochondria is localized specifically in the inner mitochondrial membrane. The existence of mRyR was further confirmed by Western blot using a RyR specific antibody, as well as by [^{3}H]ryanodine binding to isolated heart mitochondria, which showed a very high affinity ryanodine binding to mRyR (K_d=9.8 nmol L^{-1}). Ca^{2+} modulated the binding of ryanodine to mRyR in a biphasic manner. The ryanodine binding was increased at pCa 5–7 and decreased at pCa 2–4. The maximum binding was observed at pCa 5.3.

Further evidence suggested that mRyR is related to skeletal muscle type 1 ryanodine receptor (RyR1) but not cardiac muscle type 2 receptor (RyR2) [18,49]. Subtype-specific antibodies detected RyR1 in mitochondria from rat and mouse hearts but not in mitochondria isolated from RyR1 knock out mice [18]. These results were also supported by the pharmacological profile showing the modulation of ryanodine binding to mRyR by Ca^{2+}, caffeine, and adenylylmethylenedi-phosphonate (AMPPCP) in isolated heart mitochondria.

The mRyR is distinguished from SR-RyR with respect to the abundance of receptors, their sensitivity to caffeine, Mg^{2+} and ruthenium red. The density of mRyR was found ~10–20 times less than that in SR-RyR. Unlike the SR-RyR, ryanodine binding to mRyR was caffeine-insensitive. In the existence of 0.33 mmol L^{-1} Mg^{2+}, ryanodine binding to mRyR is inhibited by ~50%. In contrast, up to 1 mmol L^{-1} Mg^{2+} had no inhibitory effect on ryanodine binding to cardiac SR-RyR [50–52]. In addition, ruthenium red suppressed mitochondrial ryanodine binding (IC_{50}=105 nmol L^{-1}), which is much more potent than that observed in SR-RyR (290–1000 nmol L^{-1}) [50,51]. Consistent with these observations, ruthenium red (1–5 μmol L^{-1}) blocked mitochondrial Ca^{2+} uptake without much effect on SR Ca^{2+} release in chemically skinned cardiomyocyte [53]. All above evidence also indicates that mRyR1 has pharmacological properties similar to RyR1 but not RyR2 [18].

In response to extra mitochondrial Ca^{2+} pulse, mRyR transports Ca^{2+} rapidly. The uptake peaked within the shortest sampling interval of one image frame used (250 ms) [18]. In the presence of 10 or 100 μmol L^{-1} ryanodine, mitochondrial Ca^{2+} uptake was suppressed by 40.2%±1.9% and 60%±2.7% respectively. Similar effect was observed using dantrolene, a compound that inhibited the skeletal muscle SR-RyR and therefore Ca^{2+} release [54].

Further characterization of mRyR was done through the reconstitution of sucrose-purified mitochondrial fractions into lipid bilayers. The reconstituted channels exhibited characteristics of reconstituted RyRs. (i) The reconstituted channels yielded large conductance (500–800 pS) [17]. (ii) With the cytosolic [Ca^{2+}] changing from 5 to 50 μmol L^{-1}, both bursting frequency and mean open time of the channel were increased, indicating mRyR activation. (iii) The channels were locked into a long-lived subconductance state by low concentrations of ryanodine and completely inhibited by higher concentration of ryanodine. (iv) Impera toxin A, a high affinity RyR1 modulator, activated mRyR by promoting subconductance gating. Finally, the channels were not related to the mPTP because either cyclosporine A or bongkrekic acid had no effect.

Recently we conducted single channel characterization of the mRyR in heart by directly patch-clamping mitoplasts [13]. Patch-clamping mitoplasts allowed characterization of the native ion channel activity, which provided direct evidence on the existence of mRyR in the mitochondrial inner membrane. Among the observed four distinct channel conductances (100, 225, 700 and 1000 pS in symmetrical 150 mmol L^{-1} CsCl), the 225 pS cation-selective channel exhibited unique biophysical and pharmacological properties that distinguished it from the other large conductance ion channel activities previously described in heart mitochondria. This novel channel had multiple sub-conductance states and was blocked by ruthenium red and ryanodine, known inhibitors of ryanodine receptors. As expected the channels was modulated by ryanodine in a concentration dependent manner with lower concentration (10 μmol L^{-1}) activated while higher concentration (⩾100 μmol L^{-1}) blocked the activity. These results suggest that this novel 225 pS channel in the inner mitochondrial membrane represents native mRyR channel activity.

In summary, mRyR takes up cytosolic Ca^{2+} effectively in the physiological range. The Ca^{2+}-dependency of [^{3}H]ryanodine binding was bell-shaped. At low μmol L^{-1} concentrations, mRyR became activated. At higher concentrations (>50 μmol L^{-1}), which are the ranges of [Ca^{2+}] that favor activation of the Ca^{2+} uniporter, mRyR is inactivated. Therefore during cytosolic Ca^{2+} transients when the concentration between ER/SR and neighboring mitochondria is 1–100 μmol L^{-1}, mRyR becomes activated as soon as Ca^{2+}

is released from SR. The inactivation of mRyR by increased local Ca^{2+} afterwards may actually serve as protection from Ca^{2+}-overload-induced opening of mPTP. More importantly, the high velocity of Ca^{2+} uptake (250 ms) makes mRyR a perfect candidate to regulate Ca^{2+}-induced ATP generation in cardiac muscle on a beat to beat basis. Therefore activation of mRyR connects cardiac excitation-contraction coupling with mitochondrial energy metabolism during physiological Ca^{2+} oscillations.

4 Letm1 Ca^{2+}/H^+ antiporter

Most recently, three candidate proteins are proposed governing Ca^{2+} transport across mitochondrial inner membrane [21,22,43]. Letm 1 is one of them and the other two will be discussed as other Ca^{2+} influx mechanisms. In a genome-wide *Drosophila* RNA interference (RNAi) screen, Letm1, originally known as a K^+/H^+ exchanger [55,56], was found to mediate mitochondria Ca^{2+} and H^+ transport. Letm1 transports Ca^{2+} in and out of mitochondria in a Ca^{2+} and pH gradient-dependent manner. The Ca^{2+} uptake through Letm1 is more energy preserving than other Ca^{2+} uptake mechanisms because one Ca^{2+} uptake is coupled with one H^+ extrusion. Knockdown of Letm1, interestingly, abolished the initial fast mitochondrial Ca^{2+} uptake by histamine but exhibited sustained Ca^{2+} increase due to inhibition Ca^{2+} efflux after Ca^{2+} overload. Along with the mitochondrial Ca^{2+} change, the matrix pH showed initial alkalization followed by acidification, suggesting the duel role of Letm1 as a Ca^{2+}/H^+ exchanger during physiological agonist induced Ca^{2+} transient. Reconstitution of purified Letm1 in liposomes exhibited pH-dependent Ca^{2+} transport, which is completely abolished by nonspecific inhibitors, ruthenium red and Ru360, and partial inhibition by CGP-37157, an inhibitor of mitochondrial Na^+/Ca^+ exchanger. The calculated K_m and V_{max} of Ca^{2+} transport were 137 nmol L^{-1} Ca^{2+}/μg protein and 4.2 nmol L^{-1} Ca^{2+}/μg protein per second (~1700 ions per second) using Michaelis-Menten assumption, respectively. Letm1 was previously found to be related to Wolf-Hirshhorn syndrome characterized by neurological disorders such as mental retardation and seizure [57,58].

5 Other Ca^{2+} influx mechanisms

It has been reported that uncoupled proteins UCP2 and UCP3 are mitochondrial Ca^{2+} uniporters [20]. However, there were studies showing that dsRNAs against *Drosophila* mitochondrial UCPs did not affect $[Ca^{2+}]_m$ and $[H^+]_m$ [21]. mCa1 and mCa2 are the other two recently identified Ca^{2+} channels [59]. mCa1 and mCa2 are both voltage-dependent and highly selective for Ca^{2+}. They have maximal conductance at 105 mmol L^{-1} $[Ca^{2+}]$, and half saturation at 15.1 and 19.6 mmol L^{-1} $[Ca^{2+}]$, respectively. Interestingly, mCa1 and mCa2 have different gating parameters. mCa1 channels exhibited higher single-channel amplitude, shorter opening time, a lower open probability (P_O=0.053), and multiple subconductance states (10.1, 16.5, and 21.3 pS). On the other hand, mCa2 channels exhibited a lower single channel conductance (7.67 pS) and are insensitive to ruthenium 360. Like MCU, both mCa1 and mCa2 were activated by spermine. However, mCa2 was only partially inhibited by μmol L^{-1} concentration of Ru360. These channels were considered critical for cardiac function because in the failing hearts, mCa1 and mCa2 had decreased P_O and prolonged closed times. These results support the idea that impaired functions of these channels are accountable for the reduced mitochondrial Ca^{2+} uptake in heart failure.

6 Conclusion

Intracellular Ca^{2+} is vital for cell physiology. Disruption of Ca^{2+} homeostasis leads to human diseases such as heart failure, neuron-degeneration, and diabetes [59–61]. The role of mitochondrial Ca^{2+} transport in responding to physiological Ca^{2+} pulses is to regulate energy production and shape the amplitude and duration of Ca^{2+} transients in various micro-domains for cell signaling. In addition, recent evidence has also pointed out the potential role of mitochondrial Ca^{2+} in regulating mitochondrial fission, fusion, movement, and ROS generation.

Ca^{2+} transport across mitochondrial inner membrane is a highly synchronized process. Mitochondria localized in close proximity to the Ca^{2+} micro-domains sense and take up Ca^{2+} via multiple channels and pathways. MCU, Letm1, RaM and mRyR each play a role via their unique characteristics in Ca^{2+} affinity, kinetics and pharmacological properties. To ensure an effective intracellular Ca^{2+} dynamics, all Ca^{2+} transport proteins have to work in coordination. This precise coordination is particularly important for mitochondria to orchestrate signaling, energy metabolism, ROS generation and cell death. Further studies on the structure and function of these channels in mitochondrial inner membrane will advance our knowledge on the role of mitochondria in normal physiology and diseases.

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